

**ENHANCEMENT OF THE STABILITY OF OLIGONUCLEOTIDES
COMPRISING PHOSPHOROTHIOATE LINKAGES
BY ADDITION OF WATER-SOLUBLE ANTIOXIDANTS**

FIELD OF THE INVENTION

The present invention relates to compositions and methods for enhancing the stability of oligonucleotide formulations. More particularly, the invention relates to the addition of antioxidants which partition into the aqueous phase of a bi- or multi-phasic topical formulation to prevent desulfurization of phosphorothioate internucleoside linkages.

BACKGROUND OF THE INVENTION

Advances in the field of biotechnology have led to significant advances in the treatment of diseases such as cancer, genetic diseases, arthritis and AIDS that were previously difficult to treat. Many such advances involve the administration of oligonucleotides and other nucleic acids to a subject, particularly a human subject. The administration of such molecules via parenteral routes has been shown to be effective for the treatment of diseases and/or disorders. See, *e.g.*, Draper *et al.*, U.S. Patent No. 5,595,978, January 21, 1997, which discloses intravitreal injection as a means for the direct delivery of antisense oligonucleotides to the vitreous humor of the mammalian eye. See also, Robertson, *Nature Biotechnology*, 1997, 15, 209, and *Genetic Engineering News*, 1997, 15, 1, each of which discuss the treatment of Crohn's disease via intravenous infusions of antisense oligonucleotides.

Antisense oligonucleotides are useful in the treatment of many disorders, including cancer, inflammatory diseases and metabolic diseases (see, *e.g.*, PCT WO00/20432, PCT WO00/20635, PCT WO94/05813, U.S. Patent Nos. 6,214,986, 6,174,870 and 6,174,868). Oligonucleotides which comprise one or more

phosphorothioate linkages are known to be more stable to degradation by nucleases and to support an RNase H mode of cleavage of target RNA. However, impurities in oligonucleotide formulations, such as peroxide radicals generated from excipients, may lead to desulfurization. In this process, phosphorothioate linkages are converted to phosphodiester linkages that are much less nuclease resistant and do not support cleavage by RNase H. The resulting oligonucleotides are not suitable for therapeutic use because of their instability *in vivo*. Thus, there is a need for a method of reducing desulfurization in oligonucleotide formulations, particularly topical formulations. The present invention addresses this need.

SUMMARY OF THE INVENTION

One embodiment of the present invention is a biphasic or multiphasic formulation comprising an oligonucleotide or bioequivalent thereof which comprises one or more phosphorothioate linkages and an antioxidant that partitions into the aqueous phase of the formulation. Preferably, the oligonucleotide or bioequivalent thereof comprises one or more base modifications. In one aspect of this preferred embodiment, the oligonucleotide or bioequivalent thereof comprises one or more modified internucleoside linkages in addition to the one or more phosphorothioate linkages. Advantageously, the oligonucleotide or bioequivalent thereof comprises one or more sugar modifications. Preferably, the sugar modification is a 2'-methoxyethoxy modification. Preferably, the antioxidant is cysteine, glutathione, α -lipoic acid, a 2-mercapto-5-benzimidazole salt or a 2-mercaptoethanesulfonic acid salt. In one aspect of this preferred embodiment, the oligonucleotide is a ribozyme, aptamer or antisense oligonucleotide.

The present invention also provides a method of preventing desulfurization of an oligonucleotide or bioequivalent thereof comprising one or more phosphorothioate linkages in a bi-phasic or multi-phasic formulation, comprising including in the formulation an antioxidant which partitions into the aqueous phase of the formulation. Preferably, the oligonucleotide or bioequivalent thereof comprises one or more base modifications. In one aspect of this preferred embodiment, the oligonucleotide or bioequivalent thereof comprises one or more modified internucleoside linkages.

Preferably, the antisense oligonucleotide or bioequivalent thereof comprises one or more sugar modifications. Advantageously, the sugar modification is a 2-methoxyethoxy.

Preferably, the antioxidant is cysteine, glutathione, α -lipoic acid, a 2-mercapto-5-benzimidazole salt or a 2-mercaptoethanesulfonic acid salt. In one aspect of this preferred embodiment, the oligonucleotide is a ribozyme, aptamer or peptide nucleic acid.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides compositions and methods for enhancing the stability of oligonucleotide compounds comprising at least one phosphorothioate linkage, preferably oligonucleotides comprising one or more phosphorothioate linkages in bi-phasic and multi-phasic pharmaceutical formulations, by the addition of antioxidants which partition into the aqueous phase of such formulations. In a preferred embodiment, the oligonucleotides are antisense oligonucleotides.

As used herein, the term mono-phasic means a composition having a single phase (either aqueous or oil phase), bi-phasic means a composition having an aqueous and an oil phase, and multi-phasic means a composition having an aqueous phase, an oil phase, and at least one additional aqueous and/or oil phase. These formulations include topical formulations such as creams, lotions, ointments, salves, gels, pastes; oral formulations such as tablets, capsules and gelcaps; parenteral formulations such as solutions for intravenous, subcutaneous and intramuscular administration and rectal formulations such as enemas and suppositories.

Although several conventional antioxidants (t-butylmethoxyphenols, t-butylmethylphenols and vitamin E) were found to inhibit desulfurization of an antisense oligonucleotide comprising phosphorothioate linkages, they were ineffective at inhibiting desulfurization in a bi-phasic cream formulation. However, antioxidants which partition into the aqueous phase (L-cysteine, glutathione, α -lipoic acid, 2-mercaptobenzimidazole sulfonic acid, sodium salt) unexpectedly inhibited desulfurization in a bi-phasic cream formulation. Although only certain antioxidants that partition into the aqueous phase of these cream formulations are exemplified herein, the use of any such antioxidant is within the scope of the present invention. Similarly, the examples presented herein which discuss

bi-phasic cream formulations are meant only to be merely illustrative, and do not limit the invention to such formulations. Any bi-phasic or multi-phasic formulation which comprises one or more antioxidants which partition into the aqueous phase are contemplated for use in the present invention.

In a preferred embodiment, the antioxidant amounts in the formulation are between about 0.01 and 100 mg, more preferably between about 0.1 and 50 mg, and most preferably between about 0.5 and 25 mg.

The compositions of the invention may also include penetration enhancers. Penetration enhancers include, but are not limited to, members of molecular classes such as surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactant molecules. (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Carriers are inert molecules that may be included in the compositions of the present invention to interfere with processes that lead to reduction in the levels of bioavailable drug.

In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of oligonucleotides through the alimentary mucosa and other epithelial membranes is enhanced. In addition to bile salts and fatty acids, surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and perfluorochemical emulsions, such as FC-43 (Takahashi *et al.*, *J. Pharm. Pharmacol.*, 1988, 40, 252).

Fatty acids and their derivatives which act as penetration enhancers and may be used in compositions of the present invention include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-*rac*-glycerol), dilaurin, caprylic acid, arachidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines and mono- and di-glycerides thereof and/or physiologically acceptable salts thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate,

linoleate, etc.) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; El-Hariri *et al.*, *J. Pharm. Pharmacol.*, 1992, 44, 651).

A variety of bile salts also function as penetration enhancers to facilitate the uptake and bioavailability of drugs. The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (CDCA, sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; Yamamoto *et al.*, *J. Pharm. Exp. Ther.*, 1992, 263, 25; Yamashita *et al.*, *J. Pharm. Sci.*, 1990, 79, 579).

In a particular embodiment, penetration enhancers useful in the present invention are mixtures of penetration enhancing compounds. For example, a particularly preferred penetration enhancer is a mixture of UDCA (and/or CDCA) with capric and/or lauric acids or salts thereof e.g. sodium. Such mixtures are useful for enhancing the delivery of biologically active substances across mucosal membranes, in particular intestinal mucosa. Preferred penetration enhancer mixtures comprise about 5-95% of bile acid or salt(s) UDCA and/or CDCA with 5-95% capric and/or lauric acid. Particularly

preferred are mixtures of the sodium salts of UDCA, capric acid and lauric acid in a ratio of about 1:2:2 respectively.

Chelating agents, as used in connection with the present invention, can be defined to be compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the alimentary and other mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, 1993, 618, 315).

Chelating agents of the invention include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines)(Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1; Buur *et al.*, *J. Control Rel.*, 1990, 14, 43).

As used herein, non-chelating non-surfactant penetration enhancers may be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary and other mucosal membranes (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1). This class of penetration enhancers includes, but is not limited to, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, *J. Pharm. Pharmacol.*, 1987, 39, 621).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al.*, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), can be used

The oral pharmaceutical formulation into which the populations of carrier particles are incorporated may be, for example, a capsule, tablet, compression coated tablet or bilayer tablet. In a preferred embodiment, these formulations comprise an enteric outer coating that resists degradation in the stomach and dissolves in the intestinal lumen. In a preferred embodiment, the formulation comprises an enteric material effective in protecting the nucleic acid from pH extremes of the stomach, or in releasing the nucleic acid over time to optimize the delivery thereof to a particular mucosal site. Enteric materials for acid-resistant tablets, capsules and caplets are known in the art and typically include acetate phthalate, propylene glycol, sorbitan monoleate, cellulose acetate phthalate (CAP), cellulose acetate trimellitate, hydroxypropyl methyl cellulose phthalate (HPMCP), methacrylates, chitosan, guar gum, pectin, locust bean gum and polyethylene glycol (PEG). One particularly useful type of methacrylate are the EUDRAGITS™. These are anionic polymers that are water-impermeable at low pH, but become ionized and dissolve at intestinal pH. EUDRAGITS™ L100 and S100 are copolymers of methacrylic acid and methyl methacrylate.

Enteric materials may be incorporated within the dosage form or may be a coating substantially covering the entire surface of tablets, capsules or caplets. Enteric materials may also be accompanied by plasticizers that impart flexible resiliency to the material for resisting fracturing, for example during tablet curing or aging. Plasticizers are known in the art and typically include diethyl phthalate (DEP), triacetin, dibutyl sebacate (DBS), dibutyl phthalate (DBP) and triethyl citrate (TEC).

A "pharmaceutically acceptable" component of a formulation of the invention is one which, when used together with excipients, diluents, stabilizers, preservatives and other ingredients are appropriate to the nature, composition and mode of administration of a formulation. Accordingly, it is desired to select penetration enhancers that facilitate the uptake of drugs, particularly oligonucleotides, without interfering with the activity of the drug and in a manner such that the same can be introduced into the body of an animal without unacceptable side-effects such as toxicity, irritation or allergic response.

Oligonucleotides of the present invention may be, but are not limited to, those nucleic acids bearing modified linkages, modified nucleobases, or modified sugars, and chimeric nucleic acids. Bioequivalents of oligonucleotides and other nucleic acids are also contemplated such as, but not limited to, oligonucleotide prodrugs, deletion derivatives, conjugates and salts.

The compositions of the present invention may additionally comprise other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, do not unduly interfere with the biological activities of the components of the compositions of the present invention.

In a preferred embodiment, the pharmaceutical formulations of the present invention are used to deliver oligonucleotides for use in antisense modulation of the function of DNA or messenger RNA (mRNA) encoding a protein the modulation of which is desired, and ultimately to regulate the amount of such a protein. Hybridization of an antisense oligonucleotide with its mRNA target interferes with the normal role of mRNA and causes a modulation of its function in cells. The functions of mRNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, turnover or degradation of the mRNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with mRNA function is modulation of the expression of a protein, wherein "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of the protein. In the context of the present invention, inhibition is the preferred form of modulation of gene expression.

In the context of the present invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid or deoxyribonucleic acid. This term includes

oligonucleotides composed of naturally occurring nucleobases, sugars and covalent intersugar (backbone) linkages as well as modified oligonucleotides having non-naturally-occurring portions that function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced binding to target and increased stability in the presence of nucleases.

An oligonucleotide is a polymer of repeating units generically known as nucleotides. An unmodified (naturally occurring) nucleotide has three components: (1) a nitrogenous *base* linked by one of its nitrogen atoms to (2) a 5-carbon cyclic *sugar* and (3) a *phosphate*, esterified to carbon 5 of the sugar. When incorporated into an oligonucleotide chain, the phosphate of a first nucleotide is also esterified to carbon 3 of the sugar of a second, adjacent nucleotide. The "backbone" of an unmodified oligonucleotide consists of (2) and (3), that is, sugars linked together by phosphodiester linkages between the carbon 5 (5') position of the sugar of a first nucleotide and the carbon 3 (3') position of a second, adjacent nucleotide. A "nucleoside" is the combination of (1) a nucleobase and (2) a sugar in the absence of (3) a phosphate moiety (Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pages 4-7). The backbone of an oligonucleotide positions a series of bases in a specific order; the written representation of this series of bases, which is conventionally written in 5' to 3' order, is known as a nucleotide sequence.

Oligonucleotides may comprise nucleotide sequences sufficient in identity and number to effect specific hybridization with a particular nucleic acid. Such oligonucleotides that specifically hybridize to a portion of the sense strand of a gene are commonly described as "antisense." In the context of the invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleotides. For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a

DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that an oligonucleotide need not be 100% complementary to its target DNA sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a decrease or loss of function, and there is a sufficient degree of complementarity to avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, *i.e.*, under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense oligonucleotides are commonly used as research reagents, diagnostic aids, and therapeutic agents. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes, for example to distinguish between the functions of various members of a biological pathway. This specific inhibitory effect has, therefore, been harnessed by those skilled in the art for research uses. Antisense oligonucleotides have also been used as diagnostic aids based on their specific binding or hybridization to DNA or mRNA that are present in certain disease states and due to the high degree of sensitivity that hybridization based assays and amplified assays that utilize some of polymerase chain reaction afford. The specificity and sensitivity of oligonucleotides is also harnessed by those of skill in the art for therapeutic uses. For example, the following U.S. patents demonstrate palliative, therapeutic and other methods utilizing antisense oligonucleotides. U. S. Patent No. 5,135,917 provides antisense oligonucleotides that inhibit human interleukin-1 receptor expression. U.S. Patent No.

5,098,890 is directed to antisense oligonucleotides complementary to the *c-myb* oncogene and antisense oligonucleotide therapies for certain cancerous conditions. U.S. Patent No. 5,087,617 provides methods for treating cancer patients with antisense oligonucleotides. U.S. Patent No. 5,166,195 provides oligonucleotide inhibitors of Human Immunodeficiency Virus (HIV). U.S. Patent No. 5,004,810 provides oligomers capable of hybridizing to herpes simplex virus Vmw65 mRNA and inhibiting replication. U.S. Patent No. 5,194,428 provides antisense oligonucleotides having antiviral activity against influenzavirus. U.S. Patent No. 4,806,463 provides antisense oligonucleotides and methods using them to inhibit HTLV-III replication. U.S. Patent No. 5,286,717 provides oligonucleotides having a complementary base sequence to a portion of an oncogene. U.S. Patent No. 5,276,019 and U.S. Patent No. 5,264,423 are directed to phosphorothioate oligonucleotide analogs used to prevent replication of foreign nucleic acids in cells. U.S. Patent No. 4,689,320 is directed to antisense oligonucleotides as antiviral agents specific to cytomegalovirus (CMV). U.S. Patent No. 5,098,890 provides oligonucleotides complementary to at least a portion of the mRNA transcript of the human *c-myb* gene. U.S. Patent No. 5,242,906 provides antisense oligonucleotides useful in the treatment of latent Epstein-Barr virus (EBV) infections. Other examples of antisense oligonucleotides are provided herein.

Further, oligonucleotides used in the compositions of the present invention may be directed to modify the effects of mRNAs or DNAs involved in the synthesis of proteins that regulate adhesion of white blood cells and to other cell types. The adherence of white blood cells to vascular endothelium appears to be mediated in part if not *in toto* by five cell adhesion molecules ICAM-1, ICAM-2, ELAM-1, VCAM-1 and GMP-140. Dustin and Springer, *J. Cell. Biol.* 1987, 107, 321. Such antisense oligonucleotides are designed to hybridize either directly to the mRNA or to a selected DNA portion encoding intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1, or E-selectin), and vascular cell adhesion molecule-1 (VCAM-1) as disclosed in U.S. Patents 5,514,788 (Bennett *et al.*, May 7, 1996) and 5,591,623 (Bennett *et al.*, January 7, 1997), and pending U.S. patent applications Serial Nos. 08/440,740 (filed May 12, 1995) and 09/062,416 (filed April 17, 1998). These oligonucleotides have been found

to modulate the activity of the targeted mRNA or DNA, leading to the modulation of the synthesis and metabolism of specific cell adhesion molecules, and thereby result in palliative and therapeutic effects. Inhibition of ICAM-1, VCAM-1 and ELAM-1 expression is expected to be useful for the treatment of inflammatory diseases, diseases with an inflammatory component, allograft rejection, psoriasis and other skin diseases, inflammatory bowel disease, cancers and their metastases, and viral infection. Methods of modulating cell adhesion comprising contacting the animal with an oligonucleotide composition of the present invention are provided.

Exemplary antisense compounds include the following:

ISIS 2302 is a 2'-deoxyoligonucleotide having a phosphorothioate backbone and the sequence 5'-GCC-CAA-GCT-GGC-ATC-CGT-CA-3' (SEQ ID NO:1). ISIS 2302 is targeted to the 3'-untranslated region (3'-UTR) of the human ICAM-1 gene. ISIS 2302 is described in U.S. Patents 5,514,788 and 5,591,623, hereby incorporated by reference.

ISIS 15839 is a phosphorothioate isosequence "hemimer" derivative of ISIS 2302 having the structure 5'-GCC-CAA-GCT-GGC-**ATC-CGT-CA**-3' (SEQ ID NO:1), wherein emboldened "C" residues have 5-methylcytosine (m5c) bases and wherein the emboldened, double-underlined residues further comprise a 2'-methoxyethoxy modification (other residues are 2'-deoxy). ISIS 15839 is described in co-pending U.S. Patent application Serial No. 09/062,416, filed April 17, 1998, hereby incorporated by reference.

ISIS 1939 is a 2'-deoxyoligonucleotide having a phosphorothioate backbone and the sequence 5'-CCC-CCA-CCA-CTT-CCC-CTC-TC-3' (SEQ ID NO:2). ISIS 1939 is targeted to the 3'-untranslated region (3'-UTR) of the human ICAM-1 gene. ISIS 1939 is described in U.S. Patents 5,514,788 and 5,591,623, hereby incorporated by reference.

ISIS 2302 (SEQ ID NO: 1) has been found to inhibit ICAM-1 expression in human umbilical vein cells, human lung carcinoma cells (A549), human epidermal carcinoma cells (A431), and human keratinocytes. ISIS 2302 has also demonstrated specificity for its target ICAM-1 over other potential nucleic acid targets such as HLA-A and HLA-B. ISIS 1939 (SEQ ID NO:2) and ISIS 2302 markedly reduced ICAM-1 expression, as detected by northern blot analysis to determine mRNA levels, in C8161

human melanoma cells. In an experimental metastasis assay, ISIS 2302 decreased the metastatic potential of C8161 cells, and eliminated the enhanced metastatic ability of C8161 cells resulting from TNF- α treatment. ISIS 2302 has also shown significant biological activity in animal models of inflammatory disease. The data from animal testing has revealed strong anti-inflammatory effects of ISIS 2302 in a number of inflammatory diseases including Crohn's disease, rheumatoid arthritis, psoriasis, ulcerative colitis, and kidney transplant rejection. When tested on humans, ISIS 2302 has shown good safety and activity against Crohn's disease. Further ISIS 2302 has demonstrated a statistically significant steroid-sparing effect on treated subjects such that even after five months post-treatment subjects have remained weaned from steroids and in disease remission. This is a surprising and significant finding of ISIS 2302's effects.

The oligonucleotides used in the compositions of the present invention preferably comprise from about 8 to about 30 nucleotides. It is more preferred that such oligonucleotides comprise from about 10 to about 25 nucleotides.

Antisense oligonucleotides employed in the compositions of the present invention may also be used to determine the nature, function and potential relationship of various genetic components of the body to normal or abnormal body states of animals. Heretofore, the function of a gene has been chiefly examined by the construction of loss-of-function mutations in the gene (*i.e.*, "knock-out" mutations) in an animal (*e.g.*, a transgenic mouse). Such tasks are difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock-out" mutation would produce a lethal phenotype. Moreover, the loss-of-function phenotype cannot be transiently introduced during a particular part of the animal's life cycle or disease state; the "knock-out" mutation is always present. "Antisense knockouts," that is, the selective modulation of expression of a gene by antisense oligonucleotides, rather than by direct genetic manipulation, overcomes these limitations (see, for example, Albert *et al.*, *Trends in Pharmacological Sciences*, 1994, 15, 250). In addition, some genes produce a variety of mRNA transcripts as a result of processes such as alternative splicing; a "knock-out" mutation typically removes all forms of mRNA transcripts produced from such genes and thus cannot be used to examine the biological role of a particular mRNA transcript. By

providing compositions and methods for the simple oral delivery of drugs, including oligonucleotides and other nucleic acids, the present invention overcomes these and other shortcomings.

Specific examples of some preferred modified oligonucleotides envisioned for use in the compositions of the present invention include oligonucleotides containing modified backbones or non-natural intersugar linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that have an atom (or group of atoms) other than a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their intersugar backbone, including peptide nucleic acids (PNAs) are also considered to be oligonucleotides.

Specific oligonucleotide chemical modifications are described in the following subsections. It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the following modifications may be incorporated in a single antisense compound or even in a single residue thereof, for example, at a single nucleoside within an oligonucleotide.

A. Modified Linkages: Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States Patents that teach the preparation of the above phosphorus atom containing linkages include, but are not limited to, U.S. Patents Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677;

5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; and 5,697,248, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein (i.e., oligonucleosides) have backbones that are formed by short chain alkyl or cycloalkyl intersugar linkages, mixed heteroatom and alkyl or cycloalkyl intersugar linkages, or one or more short chain heteroatomic or heterocyclic intersugar linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patents Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the intersugar linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patents Nos.

5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, 1991, 254, 1497.

Some preferred embodiments of the present invention may employ oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-\text{CH}_2\text{-NH-O-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2-$ [known as a methylene (methylimino) or MMI backbone], $-\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2-$, $-\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2-$ and $-\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2-$ [wherein the native phosphodiester backbone is represented as $-\text{O-P-O-CH}_2-$] of the above referenced U.S. Patent 5,489,677, and the amide backbones of the above referenced U.S. Patent No. 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

B. Modified Nucleobases: The oligonucleotides employed in the compositions of the present invention may additionally or alternatively comprise nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in the *Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, *Angewandte Chemie, International Edition*,

1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyl-adenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (*Id.*, pages 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patents 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/762,488, filed on December 10, 1996, also herein incorporated by reference.

C. Sugar Modifications: The oligonucleotides employed in the compositions of the present invention may additionally or alternatively comprise one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl, O-, S-, or N-alkenyl, or O, S- or N-alkynyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀ alkyl or C₂ to C₁₀ alkenyl and alkynyl. Particularly preferred are O[(CH₂)_nO]_mCH₃, O(CH₂)_nOCH₃, O(CH₂)_nNH₂, O(CH₂)_nCH₃, O(CH₂)_nONH₂, and O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, poly-alkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for

improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl) or 2'-MOE] (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78, 486), i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxy, i.e., a O(CH₂)₂ON(CH₃)₂ group, also known as 2'-DMAOE, as described in co-owned United States patent application Serial Number 09/016,520, filed on January 30, 1998, the contents of which are herein incorporated by reference.

Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugars structures include, but are not limited to, U.S. Patents Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned United States patent application 08/468,037, filed on June 5, 1995, also herein incorporated by reference.

D. Other Modifications: Additional modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. For example, one additional modification of the oligonucleotides employed in the compositions of the present invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553), cholic acid (Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1994, 4, 1053), a thioether, e.g., hexyl-

S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660, 306; Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, 1993, 3, 2765), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J.*, 1991, 10, 111; Kabanov *et al.*, *FEBS Lett.*, 1990, 259, 327; Svinarchuk *et al.*, *Biochimie*, 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36, 3651; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14, 969), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36, 3651), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264, 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277, 923).

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned, and each of which is herein incorporated by reference.

A preferred conjugate imparting improved absorption of oligonucleotides in the gut is folic acid. Accordingly, there is provided a composition for oral administration comprising an oligonucleotide and a carrier wherein said oligonucleotide is conjugated to folic acid. Folic acid (folate) may be conjugated to the 3' or 5' termini of oligonucleotides, to a nucleobase or to a 2' position of any of the sugar residues in the chain. Conjugation may be via any suitable chemical linker utilizing functional groups on the oligonucleotide and folate. Oligonucleotide-folate conjugates and methods in preparing are described in

copending United States patent applications 09/098,166 (filed June 16, 1998) and 09/275,505 (filed March 24, 1999) both incorporated herein by reference.

E. Chimeric Oligonucleotides: The present invention also includes compositions employing antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate oligodeoxynucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. RNase H-mediated target cleavage is distinct from the use of ribozymes to cleave nucleic acids.

For example, such "chimeras" may be "gapmers," i.e., oligonucleotides in which a central portion (the "gap") of the oligonucleotide serves as a substrate for, e.g., RNase H, and the 5' and 3' portions (the "wings") are modified in such a fashion so as to have greater affinity for, or stability when duplexed with, the target RNA molecule but are unable to support nuclease activity (e.g., 2'-fluoro- or 2'-methoxyethoxy- substituted). Other chimeras include "hemimers," that is, oligonucleotides in which the 5' portion of the oligonucleotide serves as a substrate for, e.g., RNase H, whereas the 3' portion is modified in such a fashion so as to have greater affinity for, or stability when duplexed with, the

target RNA molecule but is unable to support nuclease activity (*e.g.*, 2'-fluoro- or 2'-methoxyethoxy- substituted), or *vice-versa*.

A number of chemical modifications to oligonucleotides that confer greater oligonucleotide:RNA duplex stability have been described by Freier *et al.* (*Nucl. Acids Res.*, 1997, 25, 4429). Such modifications are preferred for the RNase H-refractory portions of chimeric oligonucleotides and may generally be used to enhance the affinity of an antisense compound for a target RNA.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patents Nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, certain of which are commonly owned, and each of which is herein incorporated by reference, and commonly owned and allowed United States patent application serial number 08/465,880, filed on June 6, 1995, also herein incorporated by reference.

The present invention also includes compositions employing oligonucleotides that are substantially chirally pure with regard to particular positions within the oligonucleotides. Examples of substantially chirally pure oligonucleotides include, but are not limited to, those having phosphorothioate linkages that are at least 75% Sp or Rp (Cook *et al.*, U.S. Patent No. 5,587,361) and those having substantially chirally pure (Sp or Rp) alkylphosphonate, phosphoramidate or phosphotriester linkages (Cook, U.S. Patents Nos. 5,212,295 and 5,521,302).

The present invention further encompasses compositions employing ribozymes. Synthetic RNA molecules and derivatives thereof that catalyze highly specific endoribonuclease activities are known as ribozymes. (See, generally, U.S. Patent Nos. 5,543,508 and 5,545,729) The cleavage reactions are catalyzed by the RNA molecules themselves. In naturally occurring RNA molecules, the sites of self-catalyzed cleavage are located within highly conserved regions of RNA secondary structure (Buzayan *et al.*, *Proc.*

Natl. Acad. Sci. U.S.A., 1986, 83, 8859; Forster *et al.*, *Cell*, 1987, 50, 9). Naturally occurring autocatalytic RNA molecules have been modified to generate ribozymes which can be targeted to a particular cellular or pathogenic RNA molecule with a high degree of specificity. Thus, ribozymes serve the same general purpose as antisense oligonucleotides (*i.e.*, modulation of expression of a specific gene) and, like oligonucleotides, are nucleic acids possessing significant portions of single-strandedness. That is, ribozymes have substantial chemical and functional identity with oligonucleotides and are thus considered to be equivalents for purposes of the present invention.

Other biologically active oligonucleotides may be formulated in the compositions of the invention and used for therapeutic, palliative or prophylactic purposes according to the methods of the invention. Such other biologically active oligonucleotides include, but are not limited to, antisense compounds including, *inter alia*, antisense oligonucleotides, antisense PNAs and ribozymes (described *supra*) and EGSs, as well as aptamers and molecular decoys (described *infra*).

Sequences that recruit RNase P are known as External Guide Sequences, hence the abbreviation "EGS." EGSs are antisense compounds that direct of an endogenous nuclease (RNase P) to a targeted nucleic acid (Forster *et al.*, *Science*, 1990, 249, 783; Guerrier-Takada *et al.*, *Proc. Natl. Acad. Sci. USA*, 1997, 94, 8468).

Antisense compounds may alternatively or additionally comprise a synthetic moiety having nuclease activity covalently linked to an oligonucleotide having an antisense sequence instead of relying upon recruitment of an endogenous nuclease. Synthetic moieties having nuclease activity include, but are not limited to, enzymatic RNAs (as in ribozymes), lanthanide ion complexes, and the like (Haseloff *et al.*, *Nature*, 1988, 334, 585; Baker *et al.*, *J. Am. Chem. Soc.*, 1997, 119, 8749).

Aptamers are single-stranded oligonucleotides that bind specific ligands via a mechanism other than Watson-Crick base pairing. Aptamers are typically targeted to, *e.g.*, a protein and are not designed to bind to a nucleic acid (Ellington *et al.*, *Nature*, 1990, 346, 818).

Molecular decoys are short double-stranded nucleic acids (including single-stranded nucleic acids designed to "fold back" on themselves) that mimic a site on a

nucleic acid to which a factor, such as a protein, binds. Such decoys are expected to competitively inhibit the factor; that is, because the factor molecules are bound to an excess of the decoy, the concentration of factor bound to the cellular site corresponding to the decoy decreases, with resulting therapeutic, palliative or prophylactic effects. Methods of identifying and constructing nucleic acid decoy molecules are described in, e.g., U.S. Patent No. 5,716,780.

Another type of bioactive oligonucleotide is an RNA-DNA hybrid molecule that can direct gene conversion of an endogenous nucleic acid (Cole-Strauss *et al.*, *Science*, 1996, 273, 1386).

Examples of specific oligonucleotides and the target genes to which they inhibit, which may be employed in formulations of the present invention include:

ISIS-2302	GCCCA AGCTG GCATC CGTCA	(SEQ ID NO:1)	ICAM-1
ISIS-15839	<u>GCCCA</u> AGCTG <u>GCATC</u> <u>CGTCA</u>	(SEQ ID NO:1)	ICAM-1
ISIS-1939	CCCCC ACCAC TTCCC CTCTC	(SEQ ID NO:2)	ICAM-1
ISIS-2922	GCGTT TGCTC TTCTT CTTGC G	(SEQ ID NO:48)	HCMV
ISIS-13312	<u>GCGTT</u> <u>TGCTC</u> <u>TTCTT</u> <u>CTTGC</u> G	(SEQ ID NO:48)	HCMV
ISIS-3521	GTTCT CGCTG GTGAG TTTCA	(SEQ ID NO:49)	PKC α
ISIS-9605	<u>GTTCT</u> <u>CGCTG</u> GTGAG <u>TTTCA</u>	(SEQ ID NO:49)	PKC α
ISIS-9606	<u>GTTCT</u> <u>CGCTG</u> GTGAG <u>TTTCA</u>	(SEQ ID NO:49)	PKC α
ISIS-14859	AACTT GTG <u>CT</u> <u>TGCTC</u>	(SEQ ID NO:50)	PKC α
ISIS-2503	TCCGT CATCG CTCCT CAGGG	(SEQ ID NO:16)	Ha-ras
ISIS-5132	TCCCG CCTGT GACAT GCATT	(SEQ ID NO:19)	c-raf
ISIS-14803	GTGCT <u>CATGG</u> <u>TGCAC</u> GGTCT	(SEQ ID NO:51)	HCV
ISIS-28089	<u>GTGTG</u> <u>CCAGA</u> <u>CACCC</u> <u>TATCT</u>	(SEQ ID NO:52)	TNF α
ISIS-104838	<u>GCTGA</u> TTAGA GAGAG <u>GTCCC</u>	(SEQ ID NO:53)	TNF α
ISIS-2105	TTGCT TCCAT CTTCC TCGTC	(SEQ ID NO:54)	HPV

wherein (i) each oligo backbone linkage is a phosphorothioate linkage (except ISIS-9605) and (ii) each sugar is 2'-deoxy unless represented in bold font in which case it incorporates a 2'-O-methoxyethyl group and iii) underlined cytosine nucleosides incorporate a 5-methyl

substituent on their nucleobase. ISIS-9605 incorporates natural phosphodiester bonds at the first five and last five linkages with the remainder being phosphorothioate linkages.

F. Synthesis: The oligonucleotides used in the compositions of the present invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives.

1. Synthesis of oligonucleotides: Teachings regarding the synthesis of particular modified oligonucleotides may be found in the following U.S. patents or pending patent applications, each of which is commonly assigned with this application: U.S. Patents Nos. 5,138,045 and 5,218,105, drawn to polyamine conjugated oligonucleotides; U.S. Patent No. 5,212,295, drawn to monomers for the preparation of oligonucleotides having chiral phosphorus linkages; U.S. Patents Nos. 5,378,825 and 5,541,307, drawn to oligonucleotides having modified backbones; U.S. Patent No. 5,386,023, drawn to backbone modified oligonucleotides and the preparation thereof through reductive coupling; U.S. Patent No. 5,457,191, drawn to modified nucleobases based on the 3-deazapurine ring system and methods of synthesis thereof; U.S. Patent No. 5,459,255, drawn to modified nucleobases based on N-2 substituted purines; U.S. Patent No. 5,521,302, drawn to processes for preparing oligonucleotides having chiral phosphorus linkages; U.S. Patent No. 5,539,082, drawn to peptide nucleic acids; U.S. Patent No. 5,554,746, drawn to oligonucleotides having β -lactam backbones; U.S. Patent No. 5,571,902, drawn to methods and materials for the synthesis of oligonucleotides; U.S. Patent No. 5,578,718, drawn to nucleosides having alkylthio groups, wherein such groups may be used as linkers to other moieties attached at any of a variety of positions of the nucleoside; U.S. Patents Nos. 5,587,361 and 5,599,797, drawn to oligonucleotides having phosphorothioate linkages of high chiral purity; U.S. Patent No. 5,506,351, drawn to processes for the preparation of 2'-O-alkyl guanosine and related compounds, including 2,6-diaminopurine compounds; U.S. Patent No. 5,587,469, drawn to oligonucleotides

having N-2 substituted purines; U.S. Patent No. 5,587,470, drawn to oligonucleotides having 3-deazapurines; U.S. Patents Nos. 5,223,168, issued June 29, 1993, and 5,608,046, both drawn to conjugated 4'-desmethyl nucleoside analogs; U.S. Patent Nos. 5,602,240, and 5,610,289, drawn to backbone modified oligonucleotide analogs; and U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent No. 5,459,255, drawn to, *inter alia*, methods of synthesizing 2'-fluoro-oligonucleotides.

2. Bioequivalents: The compositions of the present invention encompass any pharmaceutically acceptable compound that, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to "prodrugs" and "pharmaceutically acceptable salts" of the antisense compounds of the invention and other bioequivalents.

A. Oligonucleotide Prodrugs: The oligonucleotide and nucleic acid compounds employed in the compositions of the present invention may additionally or alternatively be prepared to be delivered in a "prodrug" form. The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (*i.e.*, drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the antisense compounds may be prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 (Gosselin *et al.*, published December 9, 1993).

B. Pharmaceutically Acceptable Salts: The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the oligonucleotide and nucleic acid compounds employed in the compositions of the present invention (*i.e.*, salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto).

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, ammonium, polyamines such as spermine and spermidine, and the like. Examples of suitable amines are chlorprocaine,

choline, N,N'-dibenzylethylenediamine, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge *et al.*, "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66:1). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

During the process of oligonucleotide synthesis, nucleoside monomers are attached to the chain one at a time in a repeated series of chemical reactions such as nucleoside monomer coupling, oxidation, capping and detritylation. The stepwise yield for each nucleoside addition is above 99%. That means that less than 1% of the sequence chain failed to be generated from the nucleoside monomer addition in each step as the total results of the incomplete coupling followed by the incomplete capping, detritylation and oxidation (Smith, *Anal. Chem.*, 1988, 60, 381A). All the shorter oligonucleotides, ranging from (n-1), (n-2), *etc.*, to 1-mers (nucleotides), are present as impurities in the n-mer oligonucleotide product. Among the impurities, (n-2)-mer and shorter oligonucleotide impurities are present in very small amounts and can be easily removed by chromatographic purification (Warren *et al.*, Chapter 9 *In: Methods in Molecular Biology, Vol. 26: Protocols for Oligonucleotide Conjugates*, Agrawal, S., Ed., 1994, Humana Press Inc., Totowa, NJ, pages 233-264). However, due to the lack of chromatographic selectivity and product yield, some (n-1)-mer impurities are still present in the full-length (*i.e.*, n-mer) oligonucleotide product after the purification process. The (n-1) portion consists of the mixture of all possible single base deletion sequences relative to the n-mer parent oligonucleotide. Such (n-1) impurities can be classified as terminal deletion or internal deletion sequences, depending upon the position of the missing base (*i.e.*, either at the 5' or 3' terminus or internally). When an oligonucleotide containing single base deletion sequence impurities is used as a drug (Crooke, *Hematologic Pathology*, 1995, 9, 59), the terminal deletion sequence impurities will bind to the same target mRNA as the

full length sequence but with a slightly lower affinity. Thus, to some extent, such impurities can be considered as part of the active drug component, and are thus considered to be bioequivalents for purposes of the present invention.

Pharmaceutically acceptable organic or inorganic carrier substances suitable for oral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μm in diameter. (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199; Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245; Block, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 2, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 335; Higuchi *et al.*, in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water in oil (w/o) or of the oil in water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water in oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil in water (o/w) emulsion.

Emulsions may contain additional components in addition to the dispersed phases and the active drug that may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil in water in oil (o/w/o) and water in oil in water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285; Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting

surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group into: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, non-swelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 335; Idson, *Id.*, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethyl cellulose and carboxypropyl cellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used

preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245; Idson, *Id.*, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via

a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245; Block, *Id.*, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters,

fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides et al., *Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J. Pharm. Sci.*, 1996, 85, 138). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or oligonucleotides. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of oligonucleotides and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of oligonucleotides and nucleic acids within the gastrointestinal tract

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the oligonucleotides and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories - surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p. 92). Each of these classes has been discussed above.

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers,

micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. Further advantages are that liposomes obtained from natural phospholipids are biocompatible and biodegradable, liposomes can incorporate a wide range of water and lipid soluble drugs, liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes. Liposomes can be administered orally and in aerosols and topical applications.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include

carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in *Pharmaceutical Dosage Forms: Disperse Systems*, Vol. 1, Lieberman, Rieger and Banker, Eds., Marcel Dekker, Inc., New York, NY, 1988, p. 285).

In a preferred embodiment of the invention, one or more nucleic acids are administered via oral delivery.

Compositions for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, troches, tablets or SECs (soft elastic capsules or "caplets"). Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids, carrier substances or binders may be desirably added to such formulations. A tablet may be made by compression or molding, optionally with one or more accessory ingredients.

Compressed tablets may be prepared by compressing in a suitable machine, the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder (PVP or gums such as tragacanth, acacia, carrageenan), lubricant (e.g. stearates such as magnesium stearate), glidant (talc, colloidal silica dioxide), inert diluent, preservative, surface active or dispersing agent. Preferred binders/disintegrants include EMDEX (dextrate), PRECIROL (triglyceride), PEG, and AVICEL (cellulose). Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and

may be formulated so as to provide slow or controlled release of the active ingredients therein.

Various methods for producing formulations for alimentary delivery are well known in the art. See, generally, Nairn, Chapter 83; Block, Chapter 87; Rudnic *et al.*, Chapter 89; Porter, Chapter 90; and Longer *et al.*, Chapter 91 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990.

The compositions of this invention can be converted in a known manner into the customary formulations, such as tablets, coated tablets, pills, granules, capsules, aerosols, syrups, emulsions, suspensions and solutions, using inert, non-toxic, pharmaceutically suitable excipients or solvents. The therapeutically active compound is present in a concentration of about 0.5% to about 95% by weight of the total mixture, that is to say in amounts which are sufficient to achieve the stated dosage range. Compositions may be formulated in a conventional manner using additional pharmaceutically acceptable carriers or excipients as appropriate. Thus, the composition may be prepared by conventional means with carriers or excipients such as binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulfate). Tablets may be coated by methods well known in the art. The preparations may also contain flavoring, coloring and/or sweetening agents as appropriate.

Capsules used for oral delivery may include formulations that are well known in the art. Further, multicompartment hard capsules with control release properties as described by Digenis *et al.*, U.S. Patent No. 5,672,359, and water permeable capsules with a multi-stage drug delivery system as described by Amidon *et al.*, U.S. Patent No. 5,674,530 may also be used to formulate the compositions of the present invention.

The formulation of pharmaceutical compositions and their subsequent administration is believed to be within the skill of those in the art. Specific comments regarding the present invention are presented below.

In general, for therapeutic applications, a patient (*i.e.*, an animal, including a human) having or predisposed to a disease or disorder is administered one or more drugs, preferably nucleic acids, including oligonucleotides, in accordance with the invention in a pharmaceutically acceptable carrier in doses ranging from 0.01 ug to 100 g per kg of body weight depending on the age of the patient and the severity of the disorder or disease state being treated. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease or disorder, its severity and the overall condition of the patient, and may extend from once daily to once every 20 years. In the context of the invention, the term "treatment regimen" is meant to encompass therapeutic, palliative and prophylactic modalities. Following treatment, the patient is monitored for changes in his/her condition and for alleviation of the symptoms of the disorder or disease state. The dosage of the drug may either be increased if the patient does not respond significantly to current dosage levels, or the dose may be decreased if an alleviation of the symptoms of the disorder or disease state is observed, or if the disorder or disease state has been abated.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual drugs, and can generally be estimated based on EC₅₀ values found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 µg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. An optimal dosing schedule is used to deliver a therapeutically effective amount of the drug being administered via a particular mode of administration.

The term "therapeutically effective amount," for the purposes of the invention, refers to the amount of drug-containing formulation that is effective to achieve an intended purpose without undesirable side effects (such as toxicity, irritation or allergic

response). Although individual needs may vary, optimal ranges for effective amounts of formulations can be readily determined by one of ordinary skill in the art. Human doses can be extrapolated from animal studies (Katocs *et al.*, Chapter 27 *In: Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a formulation, which can be adjusted by one skilled in the art, will vary depending on the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy (if any) and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 *In: Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*, eds., McGraw-Hill, New York, NY, 1996).

Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the nucleic acid is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. For example, in the case of an individual known or suspected of being prone to an autoimmune or inflammatory condition, prophylactic effects may be achieved by administration of preventative doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years. In like fashion, an individual may be made less susceptible to an inflammatory condition that is expected to occur as a result of some medical treatment, *e.g.*, graft versus host disease resulting from the transplantation of cells, tissue or an organ into the individual.

Formulations for oral administration may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic carrier substances suitable for oral administration which do not deleteriously react with the drug of interest can be used. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin,

hydroxymethylcellulose, polyvinylpyrrolidone and the like. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation. Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

The pharmaceutical formulations, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

A number of bioequivalents of oligonucleotides and other nucleic acids may also be employed in accordance with the present invention. The invention therefore, also encompasses oligonucleotide and nucleic acid equivalents such as, but not limited to, prodrugs of oligonucleotides and nucleic acids, deletion derivatives, conjugates of oligonucleotides and salts.

The methods and compositions of the present invention also encompass the myriad deletion oligonucleotides, both internal and terminal deletion oligonucleotides, that are synthesized during the process of solid-phase manufacture of oligonucleotides for such deletion sequences are for all practical purposes bioequivalents. Synthetic RNA molecules and their derivatives that possess specific catalytic activities are known as ribozymes and are also considered bioequivalents of oligonucleotides for the purposes of the methods and compositions of the present invention. Also considered bioequivalents of oligonucleotides, for the purposes of the methods and compositions of the present invention, are peptide nucleic acids (PNAs) and aptamers (see, generally, Ellington *et al.*, *Nature*, 1990, 346, 818; U.S. Patent 5,523,389 (Ecker *et al.*, June 4, 1996)).

The name aptamer has been coined by Ellington and Szostak (Nature, 1990, 346, 818) for nucleic acid molecules that fit and therefore bind with significant specificity to non-nucleic acid ligands such as peptides, proteins and small molecules such as drugs and dyes. Because of these specific ligand binding properties, nucleic acids and oligonucleotides that may be classified as aptamers may be readily purified or isolated via affinity chromatography using columns that bear immobilized ligand. Aptamers may be nucleic acids that are relatively short to those that are as large as a few hundred nucleotides. For example, Ellington and Szostak have reported the discovery of RNA aptamers that are 155 nucleotides long and that bind dyes such as Cibacron Blue and Reactive Blue 4 (Ellington and Szostak, Nature, 1990, 346, 818) with very good selectivity. While RNA molecules were first referred to as aptamers, the term as used in the present invention refers to any nucleic acid or oligonucleotide that exhibits specific binding to small molecule ligands including, but not limited to, DNA, RNA, DNA derivatives and conjugates, RNA derivatives and conjugates, modified oligonucleotides, chimeric oligonucleotides, and gapmers.

In a preferred embodiment, the invention is drawn to the oral administration of a nucleic acid, such as an oligonucleotide, having biological activity, to an animal. By "having biological activity," it is meant that the nucleic acid functions to modulate the expression of one or more genes in an animal as reflected in either absolute function of the gene (such as ribozyme activity) or by production of proteins coded by such genes. In the context of this invention, "to modulate" means to either effect an increase (stimulate) or a decrease (inhibit) in the expression of a gene. Such modulation can be achieved by, for example, an antisense oligonucleotide by a variety of mechanisms known in the art, including but not limited to transcriptional arrest; effects on RNA processing (capping, polyadenylation and splicing) and transportation; enhancement or reduction of cellular degradation of the target nucleic acid; and translational arrest (Crooke *et al.*, *Exp. Opin. Ther. Patents*, 1996, 6, 1).

In an animal other than a human, the compositions and methods of the invention can be used to study the function of one or more genes in the animal. For example, antisense oligonucleotides have been systemically administered to rats in order to

study the role of the *N*-methyl-D-aspartate receptor in neuronal death, to mice in order to investigate the biological role of protein kinase C- α , and to rats in order to examine the role of the neuropeptide Y1 receptor in anxiety (Wahlestedt *et al.*, *Nature*, 1993, 363, 260; Dean *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1994, 91, 11762; and Wahlestedt *et al.*, *Science*, 1993, 259, 528, respectively). In instances where complex families of related proteins are being investigated, "antisense knockouts" (*i.e.*, inhibition of a gene by systemic administration of antisense oligonucleotides) may represent the most accurate means for examining a specific member of the family (see, generally, Albert *et al.*, *Trends Pharmacol. Sci.*, 1994, 15, 250).

As stated, the compositions and methods of the invention are useful therapeutically, *i.e.*, to provide therapeutic, palliative or prophylactic relief to an animal, including a human, having or suspected of having or of being susceptible to, a disease or disorder that is treatable in whole or in part with one or more nucleic acids. The term "disease or disorder" (1) includes any abnormal condition of an organism or part, especially as a consequence of infection, inherent weakness, environmental stress, that impairs normal physiological functioning; (2) excludes pregnancy *per se* but not autoimmune and other diseases associated with pregnancy; and (3) includes cancers and tumors. The term "having or suspected of having or of being susceptible to" indicates that the subject animal has been determined to be, or is suspected of being, at increased risk, relative to the general population of such animals, of developing a particular disease or disorder as herein defined. For example, a subject animal could have a personal and/or family medical history that includes frequent occurrences of a particular disease or disorder. As another example, a subject animal could have had such a susceptibility determined by genetic screening according to techniques known in the art (see, *e.g.*, U.S. Congress, Office of Technology Assessment, Chapter 5 *In: Genetic Monitoring and Screening in the Workplace*, OTA-BA-455, U.S. Government Printing Office, Washington, D.C., 1990, pages 75-99). The term "a disease or disorder that is treatable in whole or in part with one or more nucleic acids" refers to a disease or disorder, as herein defined, (1) the management, modulation or treatment thereof, and/or (2) therapeutic, palliative and/or prophylactic relief therefrom, can be provided via the administration of

more nucleic acids. In a preferred embodiment, such a disease or disorder is treatable in whole or in part with an antisense oligonucleotide.

EXAMPLES

The following examples illustrate the invention and are not intended to limit the same. Those skilled in the art will recognize, or be able to ascertain through routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of the present invention.

Example 1: Preparation of Oligonucleotides

A. General Synthetic Techniques: Oligonucleotides were synthesized on an automated DNA synthesizer using standard phosphoramidite chemistry with oxidation using iodine. Beta-cyanoethyldiisopropyl phosphoramidites were purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of 3*H*-1,2-benzodithiole-3-one-1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages.

The synthesis of 2'-O-methyl- (2'-methoxy-) phosphorothioate oligonucleotides is according to the procedures set forth above substituting 2'-O-methyl β-cyanoethyldiisopropyl phosphoramidites (Chemgenes, Needham, MA) for standard phosphoramidites and increasing the wait cycle after the pulse delivery of tetrazole and base to 360 seconds.

Similarly, 2'-O-propyl- (a.k.a 2'-propoxy-) phosphorothioate oligonucleotides are prepared by slight modifications of this procedure and essentially according to procedures disclosed in U.S. patent application Serial No. 08/383,666, filed February 3, 1995, which is assigned to the same assignee as the instant application and which is incorporated by reference herein.

The 2'-fluoro-phosphorothioate oligonucleotides of the invention are synthesized using 5'-dimethoxytrityl-3'-phosphoramidites and prepared as disclosed in U.S. patent application Serial No. 08/383,666, filed February 3, 1995, and U.S. Patent

5,459,255, which issued October 8, 1996, both of which are assigned to the same assignee as the instant application and which are incorporated by reference herein. The 2'-fluoro-oligonucleotides are prepared using phosphoramidite chemistry and a slight modification of the standard DNA synthesis protocol (*i.e.*, deprotection was effected using methanolic ammonia at room temperature).

PNA antisense analogs are prepared essentially as described in U.S. Patents Nos. 5,539,082 and 5,539,083, both of which (1) issued July 23, 1996, (2) are assigned to the same assignee as the instant application and (3) are incorporated by reference herein.

Oligonucleotides comprising 2,6-diaminopurine are prepared using compounds described in U.S. Patent No. 5,506,351 which issued April 9, 1996, and which is assigned to the same assignee as the instant application and incorporated by reference herein, and materials and methods described by Gaffney *et al.* (*Tetrahedron*, 1984, 40, 3), Chollet *et al.*, (*Nucl. Acids Res.*, 1988, 16, 305) and Prosnjak *et al.* (*Genomics*, 1994, 21, 490). Oligonucleotides comprising 2,6-diaminopurine can also be prepared by enzymatic means (Bailly *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, 1996, 93, 13623).

2'-Methoxyethoxy oligonucleotides of the invention are synthesized essentially according to the methods of Martin *et al.* (*Helv. Chim. Acta*, 1995, 78, 486).

B. Oligonucleotide Purification: After cleavage from the controlled pore glass (CPG) column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide, at 55°C for 18 hours, the oligonucleotides were purified by precipitation 2x from 0.5 M NaCl with 2.5 volumes of ethanol followed by further purification by reverse phase high liquid pressure chromatography (HPLC). Analytical gel electrophoresis was accomplished in 20% acrylamide, 8 M urea and 45 mM Tris-borate buffer (pH 7).

Additional oligonucleotides that may be formulated in the compositions of the invention include, for example, ribozymes, aptamers, molecular decoys, External Guide Sequences (EGSs) and peptide nucleic acids (PNAs).

A further preferred oligonucleotide modification includes 2'-dimethylamino oxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in co-owned United States patent application Serial Number 09/016,520, filed on January 30,

1998, the contents of which are herein incorporated by reference. Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the sugar group, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. The nucleosides of the oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Unsubstituted and substituted phosphodiester oligonucleotides are alternately synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates are synthesized as per the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, hereby incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively).

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Boranophosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for instance, alternating MMI and PO or PS linkages are prepared as described in U.S. Patents 5,378,825; 5,386,023; 5,489,677; 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5. They may also be prepared in accordance with U.S. Patents 5,539,082; 5,700,922, and 5,719,262, herein incorporated by reference.

A further preferred oligonucleotide modification includes 2'-dimethylamino oxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in co-owned United States patent application Serial Number 09/016,520, filed on January 30, 1998, the contents of which are herein incorporated by reference. Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-aminopropoxy (2'-OCH₂CH₂CH₂NH₂) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the sugar group, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. The nucleosides of

the oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Unsubstituted and substituted phosphodiester oligonucleotides are alternately synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

It is intended that each of the patents, applications, printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

Example 2

Effect of oil-soluble antioxidants in mono-phasic systems

Polyethyleneglycol-40-monostearate (0.5 g), a water-soluble excipient which forms peroxides in the absence of antioxidants, was heated at 65°C for 20 hours with 1.5 ml phosphate buffer, pH 7.0 and 1.5 mg phosphorothioate oligonucleotide (ISIS-2302) in the absence or presence of various oil-soluble antioxidants. Oligonucleotides were isolated by ethanol precipitation and phosphorothioate content was determined by strong anion exchange (SAX) chromatography. The results are shown in Table 1 (PS=phosphorothioate)

Table 1

Additive	Amount (mg)	Full PS content (area-%SAX)
No antioxidants		51.0
2+3 t-butyl-4-methoxyphenol (BHA)	5	71.5
2-t-butyl-4-methylphenol	5	91.6
2-t-butyl-5-methylphenol	5	89.4
2-t-butyl-6-methylphenol	5	93.4
Vitamin E	15	89.7

Phenolic antioxidants provided substantial protection of phosphorothioate oligonucleotides against desulfurization in mono-phasic systems.

Example 3

Effect of oil-soluble antioxidants in bi-phasic cream formulation

A cream formulation was prepared containing 5.0 g BRIJ 58 (polyoxyethylene[20]cetyl ether), 0.03% ISIS 2302 and different concentrations of phenolic antioxidants. The cream was heated at 40°C for 2 days and the oligonucleotide was isolated by ethanol precipitation and analyzed for full phosphorothioate (PS) content by SAX chromatography. The phosphorothioate content of the cream prior to heating was 95%. The results are shown in Table 2.

Table 2

Amount of antioxidant (mg) in 100g cream	Full PS content (%) with BHT	Full PS content (%) with Vitamin E	Full PS content (%) with BHA
0	83.9	83.9	83.9
5	85.8	84.0	80.7
10	82.1	81.9	86.9
25	80.1	80.6	81.1
50	85.4	78.6	77.7
75	84.4	78.7	77.7
100	85.2	76.3	74.6

The oil-soluble antioxidants BHT, vitamin E and BHA do not provide protection of phosphorothioate oligonucleotides against desulfurization in biphasic systems.

Example 4**Effect of water-soluble and oil-soluble antioxidants in bi-phasic cream formulation**

A cream formulation was prepared containing 5.0 g BRIJ 58 (polyoxyethylene[20]cetyl ether), 0.03% ISIS 2302 and different concentrations of water-soluble (L-cysteine, 2-mercaptobenzimidazole sulfonic acid, sodium salt (2-MBSA), α -lipoic acid)) and oil-soluble (2-t-butyl-4-methylphenol, 2-t-butyl-6-methylphenol, BHA, BHT, vitamin E) antioxidants. The cream was heated at 40°C for 6 days and the oligonucleotide was isolated by ethanol precipitation and analyzed for full PS content by SAX chromatography. The PS content of the cream prior to heating was about 95%. The results are shown in Table 3.

Table 3

Additive	Amount (mg)	Full PS content (%)
L-cysteine	0.05	89.6
L-cysteine	0.2	91.8
L-cysteine	0.4	93.6
L-cysteine	1.0	93.7
L-cysteine	6.1	93.0
L-cysteine	52.2	94.5
2-MBSA	4.8	95.5
α -lipoic acid	3.8	92.8
No additive		84.7
2-t-butyl-4-MP	5.0	83.8
2-t-butyl-6-MP	11.0	79.0
BHT	5.2	77.3
BHA	4.7	80.9
Vitamin E	7.4	83.7
Vitamin E-TPGS	7.5	84.8

Water-soluble antioxidants provided substantial protection from desulfurization in a bi-phasic cream formulation, while traditional oil-soluble antioxidants did not provide protection.

Example 5**Long-term protection of creams by antioxidants**

A cream formulation was prepared containing 5.0 g BRIJ 58 (polyoxyethylene[20]cetyl ether), 0.03% ISIS 2302 and different concentrations of phenolic antioxidants. The cream was heated at 40°C for 1 month and the oligonucleotide was isolated by ethanol precipitation and analyzed for full PS content by liquid chromatography/mass spectrometry (LC/MS) or SAX chromatography. The results are shown in Table 4.

Table 4

Additive	full PS-content
No excipients	91.3
No antioxidants	45.1
Cysteine	28.4(0.01%) 30.8(0.05%) 67.3(0.2%) 81.9(0.8%)
Glutathione	9.6(0.01%) 49.4 (0.05%), 50.9(0.2%) 80.1(0.8%)
α -lipoic acid	80.2(0.02%) 84.3(0.1%) 84.7(0.45%) 87.6(1.6%)
2-MBSA, Na salt	78.7(0.01%) 95.2(0.05%) 96.1(0.2%) 95.7(0.8%)
2-MESA ¹ , Na salt	61.2(0.01%) 71.6 (0.05%) 72.5(0.2%) 86.7(0.8%)
¹ 2-mercaptoethanesulfonic acid	

The antioxidants in Table 4, all of which partition into the aqueous phase of a bi-phasic formulation, provide substantial protection against desulfurization in a cream formulation.